RESEARCH PAPER

Structural apelin analogues: mitochondrial ROS inhibition and cardiometabolic protection in myocardial ischaemia reperfusion injury

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BACKGROUND AND PURPOSE

Mitochondria-derived oxidative stress is believed to be crucially involved in cardiac ischaemia reperfusion (I/R) injury, although currently no therapies exist that specifically target mitochondrial reactive oxygen species (ROS) production. The present study was designed to evaluate the potential effects of the structural analogues of apelin-12, an adipocyte-derived peptide, on mitochondrial ROS generation, cardiomyocyte apoptosis, and metabolic and functional recovery to myocardial I/R injury.

EXPERIMENTAL APPROACH

In cultured H9C2 cardiomyoblasts and adult cardiomyocytes, oxidative stress was induced by hypoxia reoxygenation. Isolated rat hearts were subjected to 35 min of global ischaemia and 30 min of reperfusion. Apelin-12, apelin-13 and structural apelin-12 analogues, AI and AII, were infused during 5 min prior to ischaemia.

KEY RESULTS

In cardiac cells, mitochondrial ROS production was inhibited by the structural analogues of apelin, AI and AII, in comparison with the natural peptides, apelin-12 and apelin-13. Treatment of cardiomyocytes with AI and AII decreased cell apoptosis concentration-dependently. In a rat model of I/R injury, pre-ischaemic infusion of AI and AII markedly reduced ROS formation in the myocardial effluent and attenuated cell membrane damage. Prevention of oxidative damage by AI and AII was associated with the improvement of functional and metabolic recovery after I/R in the heart.

CONCLUSIONS AND IMPLICATIONS

These data provide the evidence for the potential of the structural apelin analogues in selective reduction of mitochondrial ROS generation and myocardial apoptosis and form the basis for a promising therapeutic strategy in the treatment of oxidative stress-related heart disease.

Abbreviations

A12, apelin-12; A13, apelin-13; CF, coronary flow; CO, cardiac output; DMPO, 5,5-dimethyl-1pyrroline-N-oxide; EC, energy charge; EPR, electron paramagnetic resonance; H_2DCFDA, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate; H/R, hypoxia reoxygenation; I/R, ischaemia reperfusion; KHB, Krebs–Henseleit buffer; LVDP × HR, left ventricular developed pressure–heart rate product; MI, myocardial infarction; MitoPY1, mitochondria peroxy yellow 1; MitoSOX™ Red, red mitochondrial superoxide indicator; mPTP, mitochondrial permeability transition pore; PCr, phosphocreatine; pGluA13, pyroglutamate form of apelin-13; ROS, reactive oxygen species; ΣAN, total adenine nucleotide pool (ATP + ADP + AMP)

Tables of Links

TARGETS		LiGANDS
GPCR[a]	**Enzymes**[c]	A13, apelin-13
APJ (apelin) receptor	ACE2, angiotensin converting enzyme 2	pGluA13, Pyr1-A13
Transporters[b]	AMPK	
Na+/Ca2+ exchanger, SLC8	Endothelial NOS	
Na+/H+ exchanger, SLC9	Sirtuin 3	

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson et al., 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b,c]Alexander et al., 2013a,b,c).

Introduction

Ischaemic heart disease including myocardial infarction (MI) remains the major cause of mortality in the industrialized countries (Smith, 2001). The excessive generation of reactive oxygen species (ROS) and impaired cellular metabolism are closely linked with increased cell death and cardiac dysfunction (Korge et al., 2008; Circu and Aw, 2010). It is also reported that cardiac ischaemia reperfusion (I/R) is characterized by abnormal levels of adipocyte-derived circulating factors also known as adipokines (Kimura et al., 2009). Changes in the production of adipokines can affect key cellular pathways that serve to maintain metabolic and cardiovascular homeostasis (Schulze et al., 2003; Smith and Yellon, 2011). Although numerous adipokines have been identified (Van de Voorde et al., 2013), the effects of only a few in cardiometabolic ROS regulation have been extensively studied to date.

We have recently demonstrated that adipocyte-produced apelin plays an important role in metabolic and cardiovascular homeostasis (Dray et al., 2008; Pisarenko et al., 2010; Castan-Laurell et al., 2012; Pchejetski et al., 2012). Apelin acts as an endogenous ligand for the G-protein coupled APJ receptor. Both apelin and APJ receptors are widely distributed in most tissues including lung, heart, brain, skeletal muscle, kidney and liver. Apelin is produced as a 77 amino acid prepropeptide, which is cleaved to shorter biologically active C-terminal fragments. Apelin-13 (A13), its pyroglutamate form [Pyr][1]-A13 [pyroglutamate form of apelin-13 (pGluA13)], and apelin-12 (A12) are the most potent apelin peptides corresponding to the sequence 65–77 (Lee et al., 2006; Shin et al., 2013). These peptides possess a high affinity for APJ receptors and bioactivity in vivo and cause similar cellular effects. The apelin/APJ system plays an important role

in protection against I/R injury (Kleinz and Davenport, 2005; Smith and Yellon, 2011). Recent studies suggest that A13, pGluA13, A12 and, to a lesser extent, apelin-36 reduce infarct size and augment contractile function recovery in the heart of rodents after regional or global ischaemia (Simpkin et al., 2007; Kleinz and Baxter, 2008; Zeng et al., 2009; Rastaldo et al., 2011). Post-infarct treatment with pGluA13 decreases infarct size and attenuates cardiac tissue injury in rats in vivo (Azizi et al., 2013). Administration of exogenous A12 and A13 exerts potent positive inotropic effects on failing rat cardiac muscle and improves LV systolic function in dogs with advanced cardiac failure (Dai et al., 2006; Wang et al., 2013a).

The beneficial effects of apelin are mediated by multi-component cell signalling mechanisms that lead to inhibition of the mitochondrial permeability transition pore (mPTP) opening (Kleinz and Davenport, 2005; Simpkin et al., 2007) and activation of transmembrane Na$^+$/H$^+$ and Ca^{2+}/Na$^+$ exchange (Szokodi et al., 2002). Phosphorylation and activation of endothelial NOS are also implicated in myocardial protection afforded by apelin (Zeng et al., 2009; Rastaldo et al., 2011). We have recently demonstrated that apelin protects rat heart against I/R injury, prevents cardiac ROS-dependent hypertrophy and preserves cardiac function in cardiac remodelling (Foussal et al., 2010; Pisarenko et al., 2010). In contrast, apelin knockout animals exhibit increased myocardial injury and dramatic suppression of prosurvival pathways resulting in greater cardiac dysfunction (Wang et al., 2013b). In parallel, experiments on APJ-deficient animals show abolition of cardiovascular effects of apelin (Hashimoto et al., 2006). These data indicate that the apelin/ APJ receptor system is a critical regulator of the myocardial response to I/R injury and may represent a possible drug target. A biological rationale for the potential use of apelin as

a therapeutic agent is confirmed by its low level in patients with acute coronary syndromes and established coronary artery disease. Thus, plasma apelin concentration is considerably lower in patients with acute MI in comparison with the control group and this low level was maintained over time (Weir *et al.*, 2009; Tycinska *et al.*, 2010). These observations coincide with the finding that up-regulation of myocardial apelin mRNA during ischaemic insult is changed to its reversal in response to reperfusion in a rat model of MI (Kleinz and Baxter, 2008). Therefore, the lack of apelin bioavailability may contribute to myocardial injury caused by reperfusion and supports the use of exogenous apelin peptides for the treatment of acute coronary syndromes. However, apelin peptides are rapidly cleared from the circulation with a $t_{1/2}$ of no longer than several minutes because of hydrolysis by various peptidases including ACE2, a carboxypeptidase cleaving the C-terminal phenylalanine residue (Vickers *et al.*, 2002; Japp *et al.*, 2010). In this context, enhancement of the stability of apelin structure by chemical modification could be an option for addressing new therapeutic challenges.

In the present work, we explored the effects of synthetic structural analogues of A12 on myocardial ROS generation, metabolic status and cell apoptosis in myocardial I/R injury. Our results demonstrated that inhibition of mitochondrial ROS formation and apoptosis by synthetic A12 analogues markedly reduced myocardial susceptibility to I/R injury.

Methods

Animals

All animal care and experimental procedures conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985) and the recommendations of the French Accreditation of the Laboratory Animal Care and was approved by the local Centre National de la Recherche Scientifique ethics committee. Studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 58 animals were used in the experiments described here. Male Wistar rats weighing 310–340 g were housed in cages in groups of three, maintained at 20–30°C with a natural light–dark cycle. Rats were purchased from Pushchino Laboratory Animal Breeding (Pushchino, Moscow, Russia). All animals had free access to standard pelleted diet and tap water.

Heart isolation and perfusion

Rats were heparinized by i.p. injection (1600 IU·kg^{-1} body weight) and anaesthetized with urethane (1.3 g·kg^{-1} body weight). Hearts were excised and immediately placed into ice-cold Krebs–Henseleit bicarbonate buffer (KHB; composition, in mM; NaCl 118, KCl 4.7, CaCl$_2$ 3.0, Na$_2$ EDTA 0.5, KH$_2$PO$_4$ 1.2, MgSO$_4$ 1.2, NaHCO$_3$ 25.0 and glucose 11.0; saturated with a mixture of 95% O$_2$ and 5% CO$_2$; pH 7.4 ± 0.1 at 37°C; the buffer was passed through a 5 μm Millipore filter before use) until contraction stopped. The aorta was then cannulated and Langendorff perfusion was performed at a constant pressure equivalent to 80 cm H$_2$O for 15 min. Working perfusion was performed according to a modified method of Neely under constant left atrium pressure and aortic pressure of 20 and 100 cm H$_2$O respectively. A needle was inserted into the left ventricular (LV) cavity to register LV pressure via a Gould Statham P50 transducer, SP 1405 monitor and a Gould Brush SP 2010 recorder (Gould, Oxnard, CA, USA). The contractile function intensity index was calculated as the LV developed pressure–heart rate product (LVDP × HR), where *LVDP* is the difference between LV systolic and LV end diastolic pressure. Cardiac pump function was assessed by cardiac output (CO), the sum of aortic output and coronary flow (CF).

Experimental design

Hearts were subjected to a preliminary perfusion in the working mode with KHB for 20 min, and the steady-state values of cardiac function and CF were recorded (Figure 1A). Then they were randomly assigned to five groups, as follows.

(1) Control (n = 10). A 5 min Langendorff perfusion with KHB was performed at a constant flow rate of 4 mL·min^{-1} for 5 min. The hearts were subjected to 35 min of normothermic global ischaemia and reperfused in Langendorff mode at a flow rate of 4 mL·min^{-1} for 5 min and then in the working mode for the next 25 min.
(2) A13 (n = 10). A 5 min Langendorff perfusion was performed with KHB containing 140 μM A13 at a flow rate of 4 mL·min^{-1} for 5 min before 35 min of normothermic global ischaemia. Subsequent reperfusion protocol was the same as in control.
(3) A12 (n = 10). A 5 min Langendorff perfusion was performed with KHB containing 140 μM A12 at a flow rate of 4 mL·min^{-1} for 5 min before ischaemia. Reperfusion was performed as in control.
(4) Analogue AI (n = 10). A 5 min Langendorff perfusion was performed with KHB containing 140 μM AI at a flow rate of 4 mL·min^{-1} for 5 min prior to ischaemia. Then hearts were reperfused in the same manner as in control.
(5) Analogue AII (n = 10). A 5 min Langendorff perfusion was performed with KHB containing 140 μM AII at a flow rate of 4 mL·min^{-1} for 5 min before ischaemia. Subsequent reperfusion was the same as in control.

The myocardial effluent was collected in ice-cold tubes during both periods of Langendorff perfusion for immediate assessment of LDH activity. At the end of reperfusion, the hearts were freeze-clamped in liquid nitrogen for metabolite analysis. To determine the initial content of metabolites, the hearts were freeze-clamped in liquid nitrogen after steady state in separate series.

The optimal concentrations of peptides A13, A12, AI and AII were determined in preliminary experiments. Recovery of cardiac function was assessed using the protocol described above with the concentrations of the peptides in KHB of 70, 140 or 280 μM. Better recovery of the majority of cardiac function indices was observed with peptide concentration of 140 μM, which was used in further experiments. Figure 1B and C illustrates the effects of varying peptide concentrations in KHB on percentage recovery of CO at the end of reperfusion.
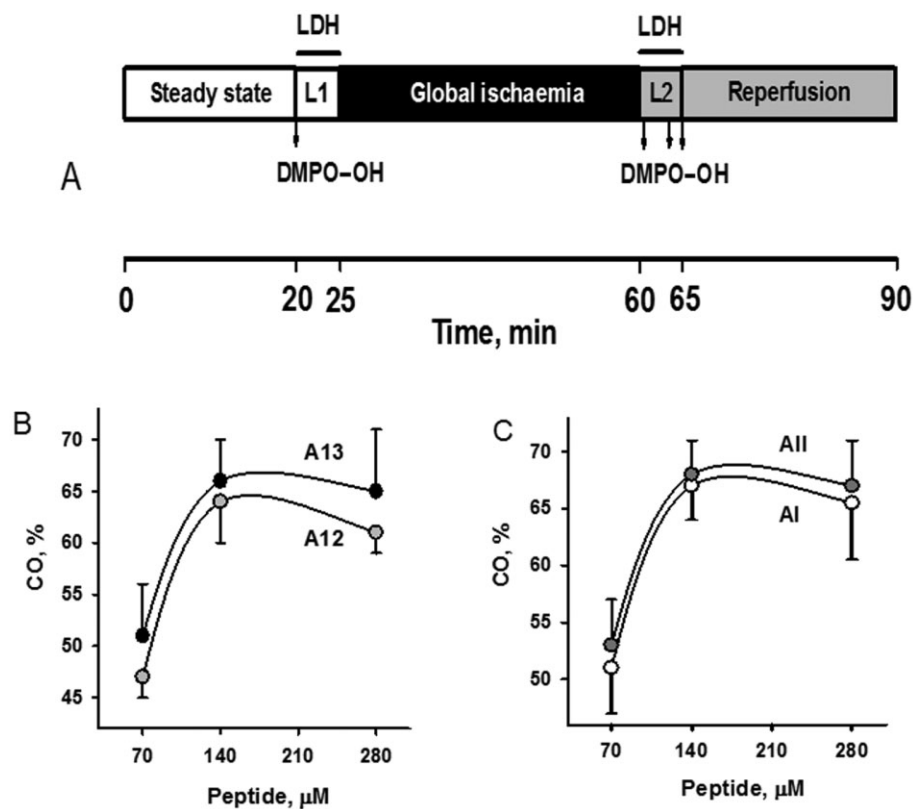
Figure 1

(A) Study design. An overview of the experiments on rat isolated hearts including perfusion protocol and determination of DMPO–OH and LDH activity in perfusate. L1 – a 5 min Langendorff perfusion at a flow rate of 4 mL·min^{-1} before global ischaemia with KHB (control) or KHB containing 140 µM apelin peptides (A13, A12, AI or AII). L2 – a 5 min Langendorff reperfusion with KHB at a flow rate of 4 mL·min^{-1} after global ischaemia. (B) Concentration-effect curve for apelin peptide A13 or A12 in KHB on CO recovery at the end of reperfusion. (C) Concentration-effect curve for the structural analogues AI or AII in KHB on CO recovery at the end of reperfusion. Recovery of CO in control was 30 ± 2% of the initial value. Data shown are the means ± SEM for 10 experiments and expressed as percentage of the initial value.

Spin trap measurements in perfusate

5,5-dimethyl-1pyrroline-N-oxide (DMPO), to give a final concentration of 100 mM, was added to aliquots of the coronary effluents collected at the end of the steady state and at 1, 3, 5 and 30 min of the reperfusion. The effluent samples were rapidly frozen and stored in liquid nitrogen until electron paramagnetic resonance (EPR) measurements. Addition of DMPO to the effluent perfusate was used to avoid improvement of cardiac function and CF by the spin trap during reperfusion (Tosaki *et al.*, 1990). Varian E-109 E X-band electron spin resonance spectrometer (Varian Inc., Palo Alto, CA, USA) was used for registration of EPR spectra of perfusate samples. EPR signals of DMPO spin adducts were recorded in a glass capillary tube at room temperature. Spectrometer settings were the following: magnetic field modulation of 0.1 mT, modulation of frequency of 100 kHz, microwave power of 10 mW and microwave frequency of 9.15 GHz. DMPO–OH spin adduct concentrations were calculated as described previously using a standard solution of the spin label 2,2,6,6-tetramethylpiperidine-N-oxyl.

Tissue processing, metabolite analysis and assay of LDH activity

Frozen tissue was quickly homogenized in cooled 6% perchloric acid (10 mL·g^{-1}) using an Ultra-Turrax T-25 homogenizer (IKA-Labortechnik, Staufen, Germany), and the homogenates were centrifuged at 2500× g for 10 min at 4°C. The supernatants were then neutralized with 5 M K$_2$CO$_3$ to pH 7.4. The protein-free extracts were centrifuged after cooling to remove the precipitated KClO$_4$. Tissue dry weights were determined by weighing a portion of the pellets after extraction with perchloric acid and drying overnight at 110°C. Concentrations of ATP, ADP, AMP, phosphocreatine (PCr) and lactate in neutralized tissue extracts were determined spectrophotometrically by enzymic methods (Bergmeyer, 1974). LDH activity in the myocardial effluent was measured according to the method of Bergmeyer and Bernt (1974) using pyruvate as substrate.

Cultured rat cardiomyocytes

Rat ventricular myocardial H9C2 cells were obtained from American Type Culture Collection (Manassas, VA, USA). Cells

Table 1

Structure of the apelin peptides and analogues studied

A13	Gln-Arg-Pro-Arg-Leu-Ser-His-Lys-Gly-Pro-Met-Pro-Phe
pGu-A13 (pGuA13)	pGlu-Arg-Pro-Arg-Leu-Ser-His-Lys-Gly-Pro-Met-Pro-Phe
A12	H-Arg-Pro-Arg-Leu-Ser-His-Lys-Gly-Pro-Met-Pro-Phe
Analogue I (AI)	**(N$^\alpha$Me)**Arg-Pro-Arg-Leu-Ser-His-Lys-Gly-Pro-**Nle**-Pro-Phe
Analogue II (AII)	**(N$^\alpha$Me)**Arg-Pro-Arg-Leu-Ser-His-Lys-Gly-Pro-**Nle**-Pro-Phe-**NH$_2$**

The peptide substitutions in the analogues are shown in bold.

were cultured in 12-well cell culture plates with DMEM (Gibco, Invitrogen, Cergy-Pontoise, France) containing 10% FBS (Invitrogen), 100 U·mL^{-1} penicillin and 100 µg·mL^{-1} streptomycin at 37°C in a humidified atmosphere of 5% CO$_2$ and were used at less than 80% of confluence.

Isolation of adult mouse cardiomyocytes

Cardiomyocytes were isolated from 10-week-old male C57BL/6J mice using enzymatic digestion and mechanical dispersion methods previously described (Zhang *et al.*, 2009). In brief, after retrograde perfusion with Ca^{2+}-free Krebs–Ringer buffer (35 mM NaCl, 4.75 mM KCl, 1.19 mM KH$_2$PO$_4$, 16 mM Na$_2$HPO$_4$, 134 mM sucrose, 25 mM Na$_2$CO$_3$, 10 mM glucose, 10 mM HEPES, pH 7.4, with NaOH) and digestion with collagenase solution (Collagenase II, 8 mg·mL^{-1}), the left ventricular myocytes were separated using a fine scalpel and scissors. After gentle trituration, cells were kept in KB solution (10 mM taurine, 70 mM glutamic acid, 25 mM KCl, 10 mM KH$_2$PO$_4$, 22 mM glucose, 0.5 mM EGTA, pH 7.2 with KOH) and studied within 6 h at room temperature.

Measurement of ROS formation in cardiomyocytes

The cellular levels of ROS were determined in isolated cardiomyocytes or H9C2 subjected to hypoxia (1% O$_2$, 5% CO$_2$) for 2 h followed by 5 min of reoxygenation (95% O$_2$, 5% CO$_2$) using H$_2$DCFDA [5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate] and red mitochondrial superoxide indicator (MitoSOX™ red). In brief, cells were washed once with 37°C HBSS/Ca/Mg buffer and incubated in 5 µM carboxy-H$_2$DCFDA, or 1 µM MitoSOX red for 30 min at 37°C followed by three washes with 37°C HBSS/Ca/Mg buffer. The fluorescence was then measured at the excitation wavelength of 488 nm for carboxy-H$_2$DCFDA and 505 nm for MitoSOX. An emission wavelength of 535 nm was used for carboxy-H$_2$DCFDA and 590 nm was used for MitoSOX. In each case, three randomly selected fields in each well were selected for examination. Levels of mitochondrial H$_2$O$_2$ were detected by measuring the intensity of the activated mitochondria peroxy yellow 1 (MitoPY1), a novel fluorescent probe for imaging H$_2$O$_2$ specifically within the mitochondria of living cells (Dickinson and Chang, 2008).

Apoptosis

TUNEL assays were performed using the kit manufacturer's protocol (Promega, Lyon, France) and as previously described

(Bianchi *et al.*, 2005). H9C2 cells were exposed to hypoxia for 16 h and fixed in 2% paraformaldehyde at –20°C. Fixed cells were incubated with TUNEL reaction mixture (terminal deoxynucleotidyl transferase and nucleotide mixture) for 1 h at 37°C. The results were expressed as the mean percentage of TUNEL-positive cells.

Data analysis

All data are presented as means ± SEM. Comparison of multiple groups was performed by two-way ANOVA followed by a Bonferroni's *post hoc* test for *in vivo* studies using GraphPad Prism version 4.00 for Windows (GraphPad Software Inc., La Jolla, CA, USA). All other statistical analyses were performed by one-way ANOVA followed by the Bonferroni *post hoc* test. Statistical significance was defined as $P < 0.05$.

Materials

The analogues of apelin, AI and AII, and A12 were synthesized by the automatic solid phase method using a 431A peptide synthesizer (Applied Biosystems, Berlingen, Germany) and Fmoc technology. In order to increase the chemical stability of A12 and its resistance to aminopeptidase cleavage, for analogue I (AI), we replaced the oxidized methionine by norleucine and included N$^\alpha$-methylarginine residue in the N-terminus of the peptide. For analogue II (AII), the N-terminal arginine residue was methylated, the methionine residue was changed to l-norleucine and the C-terminal phenylalanine was amidated. They were purified by preparative HPLC and identified by ^{1}H-NMR spectroscopy and mass spectrometry (Sidorova *et al.*, 2012). The peptide pGuA13 was a commercial product (Phoenix Pharmaceutical, Belmont, CA, USA). The structure of peptides utilized in our experiments is presented in Table 1. Other enzymes and chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Solutions were prepared using deionized water (Millipore Corp., Bedford, MA, USA).

Results

Recovery of cardiac function of isolated perfused rat heart

Effects of A13, A12, AI or AII infusion before global ischaemia on functional recovery of rat isolated perfused hearts at the end of reperfusion are shown in Table 2. In control, recovery of CF, LVDP × HR product and CO was 76, 46 and 59% as

Table 2

Effects of infusion of 140 μM A13, A12, AI or AII before global ischaemia on recovery of cardiac function and coronary flow of rat isolated perfused heart at the end of reperfusion

	Coronary flow (mL·min⁻¹)	LVDP × HR (mmHg·min⁻¹)	CO (mL)	LV diastolic pressure (mmHg)
Steady state	$17 \pm 2^\#$	$30\,897 \pm 511^\#$	$22 \pm 2^\#$	$-3 \pm 1^\#$
Control	$13 \pm 1^*$	$14\,252 \pm 588^*$	$13 \pm 1^*$	$10 \pm 1^*$
A13	$16 \pm 1^\#$	$22\,387 \pm 723^\#$	$29 \pm 1^\#$	$3 \pm 1^\#$
A12	15 ± 1	$22\,032 \pm 934^\#$	$28 \pm 2^\#$	$3 \pm 1^\#$
AI	$16 \pm 1^\#$	$22\,493 \pm 876^\#$	$29 \pm 9^\#$	$3 \pm 1^\#$
AII	$16 \pm 1^\#$	$22\,797 \pm 866^\#$	$30 \pm 1^\#$	$3 \pm 1^\#$

The values are expressed as means ± SEM for 10 experiments. $*P < 0.05$, significantly different from steady state; $^\#P < 0.05$, significantly different from control.

Table 3

Effects of infusion of 140 μM A13, A12 and analogues AI and AII before global ischaemia on myocardial metabolite contents (in μmol·g⁻¹ dry weight) and EC in rat heart at the end of reperfusion

| | Steady state | Reperfusion | | | | |
		Control	A13	A12	AI	AII
ATP	22.42 ± 2.06	$7.04 \pm 0.92^*$	$10.39 \pm 1.08^{*\#}$	$12.10 \pm 0.57^{*\#}$	$14.32 \pm 0.78^{*\#+}$	$14.59 \pm 1.03^{*\#+}$
ADP	2.80 ± 0.12	$6.13 \pm 0.44^*$	$5.91 \pm 0.36^*$	$5.66 \pm 0.22^*$	$5.02 \pm 0.32^{*+}$	$6.52 \pm 0.39^*$
AMP	0.71 ± 0.01	$3.73 \pm 0.43^*$	$3.07 \pm 0.77^*$	$1.96 \pm 0.07^*$	$1.80 \pm 0.45^*$	$2.46 \pm 0.39^*$
ΣAN	25.93 ± 1.45	$16.90 \pm 0.99^*$	$19.36 \pm 1.44^*$	$19.73 \pm 0.63^*$	21.14 ± 1.19	$23.56 \pm 0.95^\#$
EC	0.91 ± 0.02	$0.58 \pm 0.03^*$	$0.69 \pm 0.03^{*\#+}$	$0.76 \pm 0.01^{*\#+}$	$0.80 \pm 0.02^{*\#+}$	$0.75 \pm 0.02^{*\#+}$
PCr	24.30 ± 2.30	$12.69 \pm 1.59^*$	$13.26 \pm 1.63^*$	$15.48 \pm 1.17^*$	$15.85 \pm 1.77^*$	$15.20 \pm 1.41^*$
Cr	34.96 ± 2.13	$45.23 \pm 2.55^*$	$46.02 \pm 4.32^*$	$44.89 \pm 2.14^*$	43.68 ± 3.66	$44.77 \pm 3.89^*$
ΣCr	59.26 ± 1.87	57.92 ± 1.98	58.68 ± 3.16	59.65 ± 1.56	59.53 ± 3.31	60.64 ± 3.03
Lactate	1.72 ± 0.19	$6.93 \pm 1.29^*$	$1.91 \pm 0.44^\#$	$1.67 \pm 0.38^\#$	$1.86 \pm 0.48^\#$	$1.47 \pm 0.24^\#$

Values are the mean ± SEM of eight experiments. $*P < 0.05$, significantly different from steady state; $^\#P < 0.05$, significantly different from control; $^+P < 0.05$, significantly different from A13. ΣAN = ATP + ADP + AMP. ΣCr = PCr + Cr. The EC = (ATP + 0.5ADP)/ΣAN.

compared with the steady-state values, respectively; LV diastolic pressure was significantly higher than the initial value (10 ± 1 and −3 ± 1 mmHg respectively). Infusion of peptides A13, A12, AI or AII enhanced recovery of LVDP × HR product, CO and CF on average 1.6, 2.2 and 1.2 times compared with control respectively. These effects were accompanied by a reduction of LV diastolic pressure to 3 ± 1 mmHg at the end of reperfusion, thus suggesting attenuation of reperfusion contracture. No significant differences were found in the effects of the peptides on recovery of cardiac function indices or CF.

The metabolic state of isolated rat hearts after reperfusion

Changes in the myocardial metabolite contents at the end of reperfusion are compared with the steady-state values in Table 3. The control group exhibited a dramatic decrease in ATP content (to 31% of the pre-ischaemic value) and a profound build-up of ADP and AMP. The total adenine nucleo-

tide pool (ATP + ADP + AMP) (ΣAN) and the energy charge (EC) of post-ischaemic cardiomyocytes were reduced by almost 40% of the steady-state values. PCr content was less than 50% of the steady-state value, while myocardial lactate was four times higher. These shifts reflected a poor recovery of aerobic metabolism in reperfused hearts.

Pre-ischaemic infusion of peptides A13, A12, AI or AII reduced myocardial lactate content to the initial value. This effect was combined with enhanced restoration of ATP and the EC in reperfused hearts. All peptides improved preservation of the ΣAN pool compared with control; in case of analogue AII this effect was statistically significant. Analogues AI and AII preserved ATP and the EC in reperfused heart better than peptide A13. Additionally, analogue AI significantly improved ATP restoration compared with peptide A12. The peptides did not significantly affect the recovery of PCr or preservation of the ΣCr pool in reperfused hearts compared with control. These data suggest that these apelin peptides
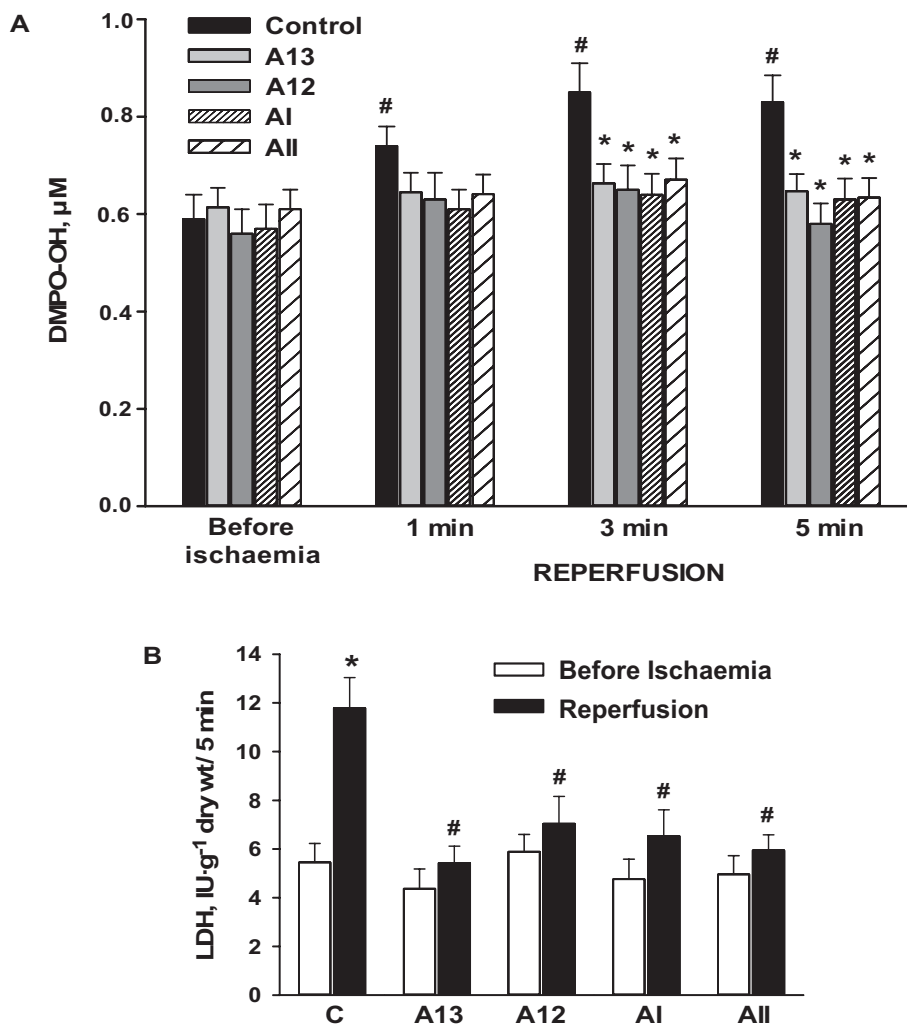
Figure 2
Effects of peptides on ROS formation and cell membrane damage during reperfusion of rat isolated heart. (A) DMPO–OH adduct concentrations in the myocardial effluent. *$P < 0.05$, significantly different from control; #$P < 0.05$, significantly different from the value before ischaemia. (B) LDH leakage from perfused rat heart before and after global ischaemia. Data shown are the mean ± SEM of six to eight experiments and expressed in $IU \cdot g^{-1}$ dry weight for 5 min Langendorff perfusion before or after global ischaemia. *$P < 0.05$, significantly different from value before ischaemia, #$P < 0.05$, significantly different from control (C) on early reperfusion.

enhanced ATP formation in reperfused hearts, stimulating glucose utilization.

DMPO–OH adduct formation in coronary effluent of isolated perfused rat heart

The effluent DMPO–OH concentrations did not differ significantly between the groups before ischaemia (Figure 2A), thus indicating that infusion of peptides does not initiate ROS formation. A significant increase in DMPO–OH concentration was observed at the first, third and fifth minutes of reperfusion in the control group, compared with the steady-state value. Pre-ischaemic administration of A13, A12, AI or AII decreased DMPO–OH formation during reperfusion compared with control. This effect was statistically significant for all peptides at the third and fifth minutes of reperfusion. In this case, DMPO–OH concentrations did not differ from the pre-ischaemic values. The DMPO–OH adduct could be

formed from a decomposition of the superoxide radical adduct DMPO–OOH or from direct trapping of OH· radicals generated in the Haber–Weiss and the Fenton reactions (Tosaki *et al.*, 1990). Thus the detection of DMPO–OH adduct in the coronary effluent did not directly reflect ROS formation in the heart and the data obtained indicated that the peptides reduced the release of ROS-generating systems and hydrogen peroxide from myocardial tissue.

Effects of peptides on LDH leakage from isolated perfused rat heart

The ability of A13, A12, AI and AII to reduce cell membrane damage was assessed by changes in LDH release into the myocardial effluent before and after global ischaemia (Figure 2B). LDH leakage did not differ significantly between the peptide groups and control for 5 min of perfusion before ischaemia. Therefore, infusion of peptides by itself did not
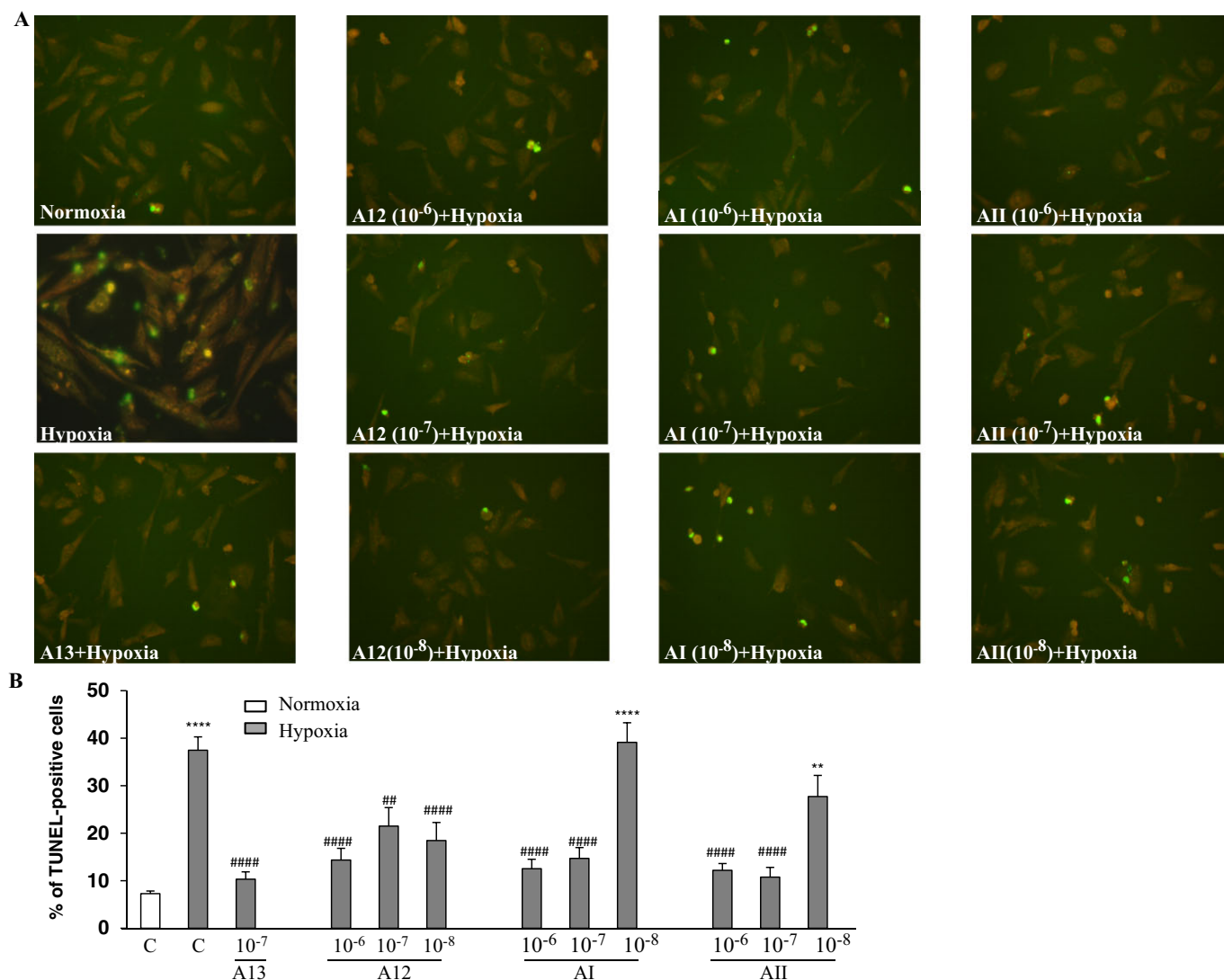
A

B

Figure 3

Concentration-dependent effects of apelin analogues on hypoxia-induced cell apoptosis. (A) Phase contrast and fluorescent images of H9C2 cells treated with apelin peptides. Apoptosis was assessed in H9C2 cells exposed to 16 h hypoxia by TUNEL assay. Cells were treated with pGluA13 (A13, 10^{-7} M), A12 (10^{-6}, 10^{-7}, 10^{-8} M), AI (10^{-6}, 10^{-7}, 10^{-8} M) and AII (10^{-6}, 10^{-7}, 10^{-8} M) 10 min before hypoxia. (B) Quantitative analysis of hypoxia-induced apoptosis in H9C2 cells. Apoptosis was evaluated by TUNEL assay and expressed as percentage of TUNEL-positive cells. Values are the means ± SEM for three experiments. **$P < 0.01$, ****$P < 0.0001$ significantly different from normoxia (N); ##$P < 0.01$, ####$P < 0.0001$, significantly different from hypoxia (H).

cause damage to the sarcolemma of non-ischaemic cardio-myocytes. The release of LDH for 5 min of reperfusion in control increased by more than twofold compared with the value before ischaemia, indicating I/R membrane damage. The post-ischaemic LDH leakage was significantly attenuated in all peptide groups compared with control suggesting fewer membrane defects.

Apoptosis

In order to determine whether apelin analogues affect cell death in pathological situation, we examined the effects of A12, AI and AII on hypoxia-induced apoptosis in H9C2 cells using the TUNEL labelling. Control H9C2 cells showed spontaneous apoptosis (7.3 ± 0.5%) when examined in normoxic

conditions (Figure 3). When subjected to hypoxia for 16 h, significant apoptosis (37.5 ± 2.8%) was observed. As shown in Figure 4, apoptotic cells had classic condensation and fragmentation of their nuclei. Assessment of TUNEL-positive cells with A12, AI and AII revealed a dose-dependent decrease in the number of apoptotic H9C2 cells (Figure 3B). As shown in Figure 4, the anti-apoptotic effects of AI and AII at concentrations of 10^{-6} to 10^{-7} M were comparable with those of A12 (10^{-7} M) and pGluA13 (10^{-7} M).

Effects of peptides on ROS formation in cardiomyocytes

To test whether apelin analogues regulate ROS homeostasis in pathophysiological conditions, we measured the effects of
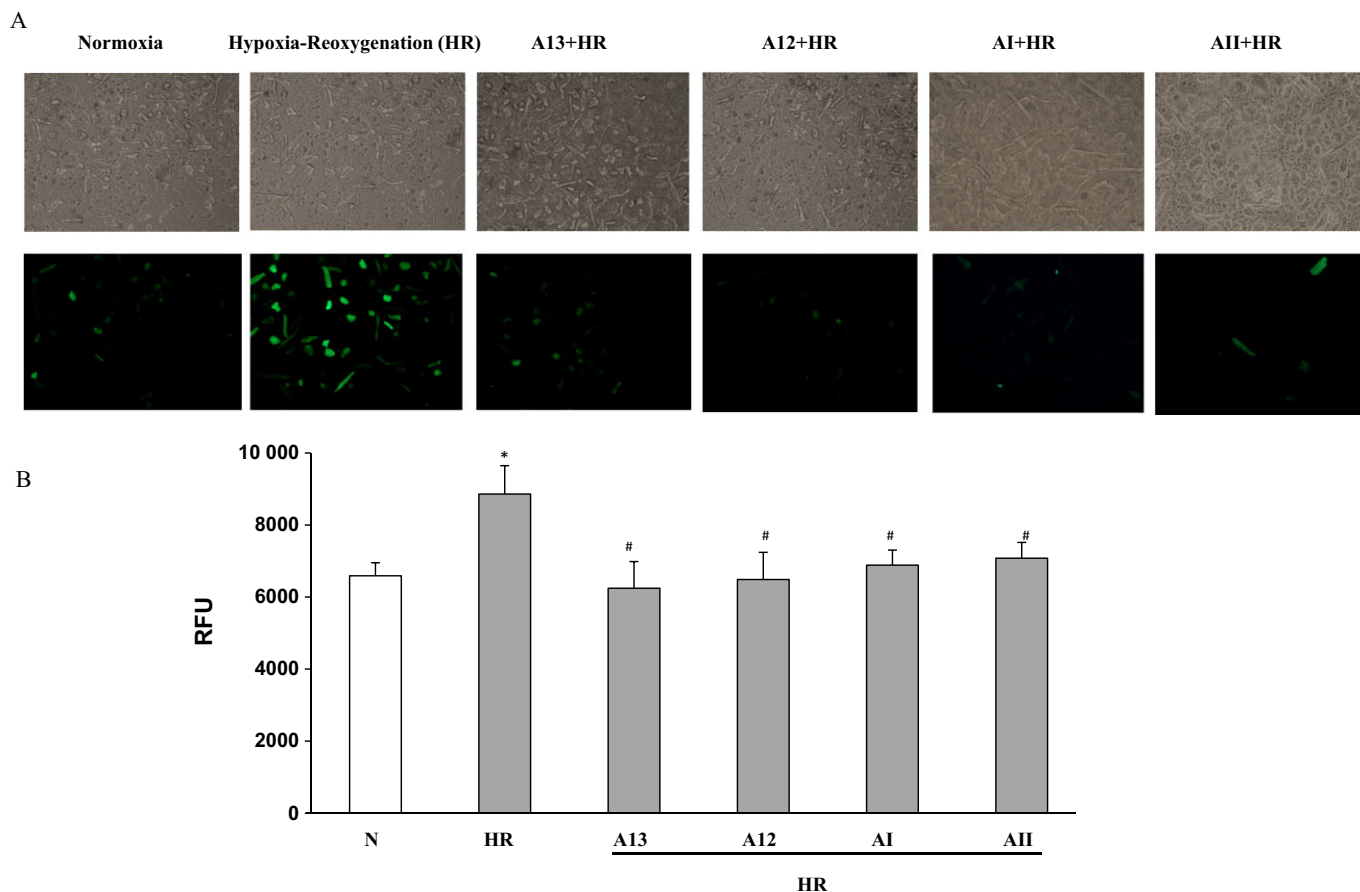
Figure 4

Effects of apelin analogues on ROS generation in cultured cardiomyocytes. (A) Representative fluorescent images of cultured cardiomyocytes cells treated with apelin peptides. Isolated mouse cardiomyocytes were subjected to hypoxia for 2 h followed by reoxygenation for 5 min to reveal the intracellular ROS production using CM-H_2DCFDA. Cells were treated with pGluA13 (A13, 10^{-7} M), A12 (10^{-7} M), AI (10^{-7} M) and AII (10^{-7} M) 10 min before hypoxia. (B) Quantitative analysis of hypoxia-induced ROS generation in cultured cardiomyocytes. Values are the means ± SEM for three experiments. *$P < 0.05$, significantly different from normoxia (N); #$P < 0.05$, significantly different from H/R.

pGluA13, A12, AI and AII on ROS generation in cells subjected to hypoxia reoxygenation (H/R) using different fluorescent ROS-sensitive probes. Analysis of dichlorodihydrofluorescein-detectable ROS revealed an increase in fluorescence intensity in cultured adult mouse cardiomyocytes subjected to H/R (Figure 4A and B). Treatment of cardiomyocytes with A12, AI and AII markedly decreased H/R-induced ROS generation. As a positive control pGluA13 (10^{-7} M) was used. Consistent with these results, confocal microscopic imaging demonstrated significantly decreased fluorescence intensity of MitoSOX Red in A12-, AI- and AII-treated H9C2 cells (Figure 5). In addition, as shown in Figure 6, both analogues AI and AII significantly attenuated H/R-stimulated fluorescence intensity of MitoPY1, a new fluorescent probe for imaging H_2O_2 level within the mitochondria, indicating the selective inhibition of mitochondrial ROS by apelin analogues.

Discussion

Myocardial ischaemia and reperfusion involves complex pathophysiological events that lead to increased oxidative stress, cardiometabolic dysfunction and cell apoptosis (Turer and Hill, 2010). Because mitochondria are key regulators for energy metabolism, ROS production and cell fate, targeting mitochondria might be a promising strategy for selective protection of cardiac cells in limiting myocardial I/R injury. In the present study, we show for the first time that the physiologically active, native peptides pGuA13 and A12 decreased mitochondrial ROS generation in cardiomyocytes subjected to hypoxia and reoxygenation. In addition, pre-ischaemic infusion of A13 and A12 reduced functional and metabolic responses of isolated perfused heart to global ischaemia and reperfusion. These beneficial effects were accompanied by decreased formation of the hydroxyl radical adduct DMPO–OH and decreased LDH leakage in the myocardial effluent during the early reperfusion. We also explored the action of the novel structural analogues of A12 on mitochondrial ROS production, cardiomyocyte apoptosis and myocardial metabolic status. Our results confirm the potent inhibitory activity of the A12 analogues against mitochondrial ROS formation, ATP depletion and apoptotic cell death. These experimental data indicate the therapeutic potential of A12 analogues in attenuating oxidative and metabolic damage induced by myocardial I/R injury.
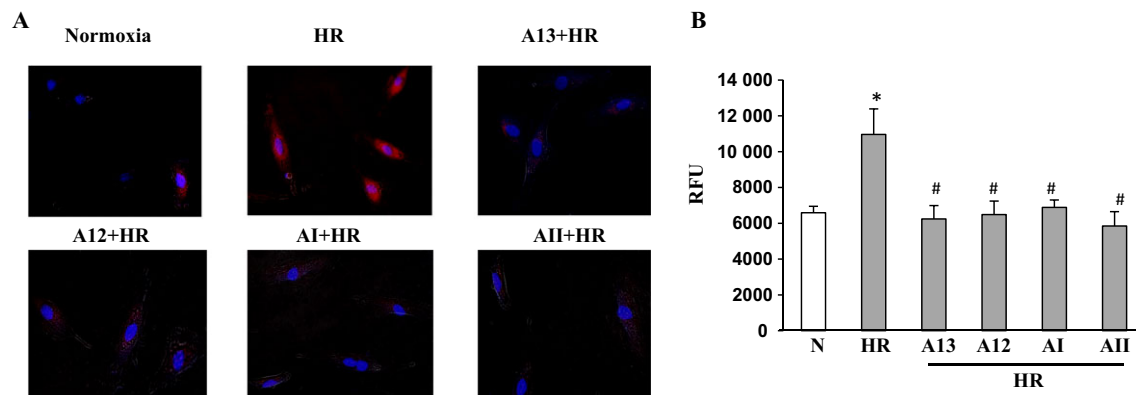
Figure 5

Effects of apelin analogues on mitochondrial superoxide generation stimulated by H/R in cultured H9C2 cells. (A) Representative confocal images of H9C2 cells showing mitochondrial MitoSOX fluorescence in H9C2 cells subjected to hypoxia for 2 h followed by reoxygenation for 5 min. Mitochondrial superoxide was detected using the fluorescent MitoSOX probe. Cells were treated with pGluA13 (A13, 10^{-7} M), A12 (10^{-7} M), AI (10^{-7} M) and AII (10^{-7} M) 10 min before hypoxia. (B) Quantitative analysis of superoxide production in H9C2 cells. Values are the means ± SEM for three experiments. *$P < 0.05$, significantly different from normoxia (N); #$P < 0.05$, significantly different from H/R.
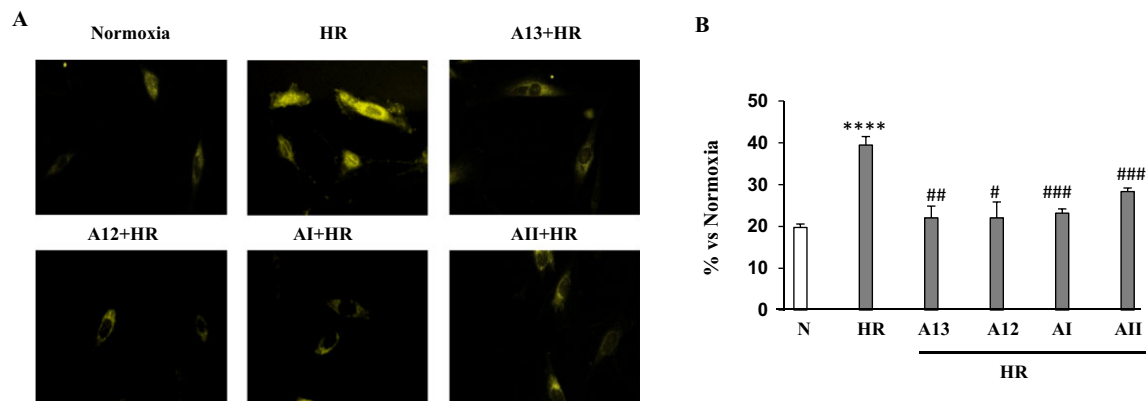

Figure 6

Effects of apelin analogues on mitochondrial H_2O_2 stimulated by H/R in cultured H9C2 cells. (A) Representative confocal images of H9C2 cells showing mitochondrial MitoPY1 fluorescence in H9C2 cells subjected to hypoxia for 2 h followed by reoxygenation for 5 min. Mitochondrial H_2O_2 was detected using the fluorescent MitoPY1 probe. Cells were treated with pGluA13 (A13, 10^{-7} M), A12 (10^{-7} M), AI (10^{-7} M) and AII (10^{-7} M) 10 min before hypoxia. (B) Quantitative analysis of H_2O_2 production in H9C2 cells. Data shown are the means ± SEM for three experiments. ****$P < 0.001$, significantly different from normoxia (N); #$P < 0.05$, ##$P < 0.01$, ###$P < 0.005$, significantly different from H/R.

The present results agreed with the earlier reports in cell culture and animal models where peptides A13, pGuA13 and A12 increased cellular survival in different models of I/R injury (Simpkin et al., 2007; Zeng et al., 2009; Zhang et al., 2009; Azizi et al., 2013). One of the principal findings of our study is that enhanced cardiomyocyte viability afforded by natural apelins was associated with decreased generation of short-lived mitochondrial ROS. This phenomenon may be related to an increase in enzymatic antioxidant activity induced by the peptides or to their direct antioxidant action. Indeed, we have previously demonstrated that pGuA13 treatment enhances myocardial catalase activity and prevents oxidative stress-linked hypertrophy in cardiomyocytes and pressure overload-induced cardiac remodelling (Foussal et al., 2010). In a recent study, we have observed stimulation of

Cu,Zn superoxide dismutase (SOD), catalase and GSH peroxidase (GSH-Px) activities by A12 in rat isolated heart during reperfusion (Pisarenko et al., 2014). However, reasons for the up-regulation of antioxidant enzymes by apelin peptides remain unexplored. In this respect, the only finding was the enhancement of catalase mRNA expression induced by pGuA13 in cultured rat cardiomyocytes (Foussal et al., 2010). Further studies are needed to evaluate the ability of these peptides per se to scavenge ROS or affect catalytic activity of antioxidant enzymes.

The potential mechanisms of reducing mitochondrial ROS production by apelin peptides are closely related to up-regulation of antioxidant enzymes. Although mitochondria express protective antioxidants, the oxidation of proteins and the accumulation of DNA lesions may contribute to the

development of oxidative stress (Ames *et al.*, 1995). In this context, activation of GSH-Px associated with the mitochondrial membrane and mitochondrial catalase and Mn SOD may be of critical importance for reducing lipid peroxides and H_2O_2 formation. Another possibility involves mobilization of the PI3K–Akt and MEK1/2–ERK1/2 salvage kinases, inhibition of the mPTP opening (Simpkin *et al.*, 2007) and protection against apoptosis. The concomitant reduction of cytochrome *c* release from the intermembrane space may favour reduction of mitochondrial $O_2^{-•}$ as the respiratory chain becomes less reduced between complex III and complex IV. Finally, there is a mechanistic link between proteins controlling mitochondrial biogenesis and mitochondrial antioxidant capacity, which could potentially be realized by apelin peptides. Apelins enhance expression of the transcriptional coactivator peroxisome proliferator-activated receptor coactivator-1 (PGC-1α) through the AMPK pathway in different cell types (Than *et al.*, 2014). PGC-1α coordinates the transcriptional activity of several nuclear transcription factors such as nuclear respiratory factors 1 and 2 and activates the genes involved in the respiratory chain, mitochondrial import machinery and transcription factors of mtDNA. Precisely because of this, apelins may regulate the induction of antioxidant defences including SODs, catalase and GSH-Px, and augment expression of the metabolic sensor NAD^+-dependent deacetylase sirtuin 3, a key regulator of the mitochondrial antioxidant system. The possibilities of such regulation of mitochondrial ROS production has stimulated great interest in targeting these pathways with apelin peptides for new treatments of cardiac dysfunction.

The cardioprotection that we observed with the natural peptides A13 and A12 was also exhibited by the structural A12 analogues, AI and AII. The present study has revealed that treatment with modified analogues of A12 improved rat cardiomyocyte survival during H/R by protecting against apoptotic cell death. We also found that both A12 analogues decreased mitochondrial ROS generation in a concentration-dependent manner in cultured cardiomyocytes subjected to H/R. Mitochondrial oxidative stress has been reported to induce mitochondrial dysfunction, promote the mPTP opening and the release of proapoptotic proteins, and subsequently lead to cell death in the form of apoptosis or necrosis (Chen and Chow, 2005; Baines, 2011). Thus, inhibition of mitochondrial superoxide formation may be of critical importance for myocardial protection with these peptides. Noteworthy, in rat isolated hearts subjected to I/R stress, we demonstrated that both AI- and AII-mediated improvement of functional and metabolic recovery was associated with reduction of ROS formation in the myocardial effluent and higher integrity of the sarcolemma. Therefore, synthesized structural analogues of A12 entirely mimic the function of native apelins in protecting compromised myocardium from irreversible injury by reducing ROS generation, apoptosis and myocardial metabolism. In addition to suppression of apoptosis by the analogue peptides AI and AII found here, we have previously shown that i.v. administration of analogue AI limited MI in rats *in vivo* and reduce activity of necrosis markers in blood plasma (Pisarenko *et al.*, 2013). Acting as autocrine or paracrine factors, apelin analogues may play a protective role in ischaemic myocardium through promoting angiogenesis and decreasing permeability of microvascular endothelial cells (Tempel *et al.*, 2012; Azizi *et al.*, 2013). Such a wide range of activity, considerably exceeding the effects observed in this study, suggests the suitability of using modified apelin for therapeutic interventions. The advantages of these novel bioactive compounds compared with the natural apelin peptides are primarily pharmacokinetic. Thus the analogue peptides are more resistant to degradation by proteolytic enzymes and consequently have a longer half-life in the circulation, along with better storage stability (Sidorova *et al.*, 2012). We have evaluated proteolytic stability of analogues AI and AII using ^{1}H-NMR techniques in model experiments with human plasma. Stability of both analogues was on average 15 times greater than that of the natural peptides A12 and A13 at the same incubation conditions (unpublished data). In accordance with this, we assume that the $t_{1/2}$ of analogue AI or AII in blood circulation may be prolonged up to 1 h. We therefore propose that chemical modification of C-terminal fragments of apelin is a useful approach to design new therapeutic tools for the treatment of cardiovascular diseases.

In conclusion, the results of this study provide the first evidence that structural analogues of apelin attenuate excessive mitochondrial ROS production and preserve myocardial metabolic status in cultured cardiomyocytes and rat isolated heart models of ischaemia and reperfusion. Further, we show that a selective inhibition of mitochondrial ROS formation by apelin analogues is associated with inhibition of apoptosis, reduction of cell membrane damage and improvement of cardiac function. Thus, these peptides can be considered as prototypes of drugs to regulate the mitochondrial oxidative stress, apoptosis and metabolic status in ischaemic heart disease.

Author contributions

O. P., A. P., P. V. and O. K. conceived and designed the experiments. V. S., I. S., Y. P., A. T. and O. K. performed the experiments. O. P., V. S., R. A. and O. K. analyzed the data. R. A. and I. S. contributed reagents/materials/analysis tools. O. K. and O. P. wrote the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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Table S1 Effects of peptide infusion before global ischaemia on recovery of LVDP (in mmHg) during reperfusion of rat isolated perfused heart.